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GRANT NUMBER DAMD17-97-1-7183

TITLE: The Role of VDA Phosphorylation in Vitamin D Induced Apoptosis

PRINCIPAL INVESTIGATOR: Carmen J. Narvaez, Ph.D.

CONTRACTING ORGANIZATION: Notre Dame University
Notre Dame, Indiana 46556-5612

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Fort Detrick, Maryland 21702-5012

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13. ABSTRACT *(Maximum 200 words)*

Vitamin D compounds are currently in clinical trials for human breast cancer and offer an alternative approach to anti-hormonal therapies for this disease. 1,25-Dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, acts through the nuclear vitamin D receptor (VDR) and is a potent negative growth regulator of breast cancer cells. Studies were initiated to examine the phosphorylation state of the VDR in relation to apoptosis. These studies involved immunoprecipitation of the VDR from MCF-7 cells treated with 1,25-D_3 or TPA, a protein kinase C activator. This technique yielded inconclusive data due to low VDR abundance coupled with non-specific antibody binding. Additional methods, including use of antibody affinity columns are now being developed for this project. To determine whether phosphorylation pathways influence vitamin D signaling downstream of the VDR, we have focused on identification of specific intracellular events involved in 1,25-D_3 mediated apoptosis such as the effects of 1,25-D_3 on mitochondrial function and caspase activity. The major findings are that 1,25-D_3 induces apoptosis in MCF-7 cells by disrupting mitochondrial function which is accomplished by translocation of Bax to mitochondria, release of cytochrome *c*, and induction of reactive oxygen species production. The effect of 1,25-D_3 signaling on mitochondria does not require caspase activation, since caspase inhibitor (zVAD.fmk) was unable to block these events. Although caspase inhibitor was able to block events downstream of mitochondria such as PARP cleavage, external display of phosphatidylserine, and DNA fragmentation, the commitment of MCF-7 cells to 1,25-D_3 mediated cell death is caspase independent.

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Carmen J. Marmay 7/23/99

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INTRODUCTION

1,25-Dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, is not only a powerful regulator of calcium homeostasis, but is a steroid hormone with important roles in cell growth and differentiation. 1,25-D_3 is a potent negative growth regulator of breast cancer cells both *in vitro* and *in vivo*. A variety of synthetic vitamin D analogs that induce breast tumor regression in animals are now undergoing clinical trials in human patients. Our lab has shown that inhibition of breast cancer cell growth in response to 1,25-D_3 involves activation of apoptosis (1,2). 1,25-D_3 acts through the nuclear vitamin D receptor (VDR), a phosphoprotein, which can be phosphorylated by protein kinase C (PKC) and casein kinase II, and modulates gene expression. The main objective of this study was to assess the phosphorylation state of the VDR and to determine how this affects specific DNA binding and transactivation ability of the VDR in MCF-7 cells in relation to apoptosis following treatment with 1,25-D_3 or TPA (a PKC activator). In addition to examining the phosphorylation state of the VDR, we investigated the 1,25-D_3 signaling pathway in order to identify specific intracellular events involved in 1,25-D_3 mediated apoptosis and to characterize events which are blocked in $MCF7^{D3Res}$ cells (a vitamin D_3-resistant variant). In particular, the effects of 1,25-D_3 mediated apoptosis on mitochondrial function and caspase activity were studied. Mitochondria play a central role in controlling cell death. Translocation of Bax from cytosol to mitochondria, release of cytochrome *c*, and the activation of caspases may initiate disruption of mitochondrial function (3,4). It may be during this mitochondrial phase that the cell makes a commitment to die. Events downstream of mitochondrial disruption are characterized by the action of caspase and nuclease activators released from mitochondria on the ultimate destruction of the cell. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells; we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis. In addition, the effects of 1,25-D_3 on $MCF\text{-}7^{D3Res}$ cells were compared to the parental MCF-7 cells to determine events that contribute to resistance. These data will help in evaluating the interactions between phosphorylation pathways and vitamin D mediated apoptosis of breast cancer cells.

Immunoprecipitation of the VDR

The main objective of this study was to assess the phosphorylation state of the VDR and to determine how this affects specific DNA binding and transactivation ability of the VDR in MCF-7 cells in relation to apoptosis following treatment with 1,25-D_3 or TPA (a PKC activator). However, technical difficulties were encountered while conducting experiments to accomplish the main objective. During immunoprecipitation with 9A7 monoclonal antibody, the heavy chain IgG (which has a similar MW as VDR) interfered with identification of the lower abundant VDR protein. Hence, the phosphorylation states of the VDR after treatment with 1,25-D_3 or TPA could not be determined using this method. To circumvent this problem, I will prepare antibody affinity columns. The 9A7 antibody will be coupled indirectly to Protein A beads via an anti-immunoglobulin antibody. Once the antibodies are bound, they will be cross-linked to protein A via a bifunctional coupling reagent, dimethylpimelimidate. In this way, during immunoprecipitation, only the VDR will be eluted whereas the 9A7 antibody remains bound to the Protein A/Sepharose. In addition, we have obtained a rabbit polyclonal VDR antibody (Santa Cruz) that will not pick up the rat heavy chain IgG when performing western blots.

Effect of TPA on VDR binding to DNA and transactivation

To characterize the effect of TPA on VDR binding to DNA, which was the second part of the main objective, nuclear extracts of MCF-7 cells treated with 1,25-D_3 or TPA were prepared in the presence of protease and phosphatase inhibitors and incubated with radiolabeled oligonucleotides corresponding to

human p21 VDRE. Extracts treated with TPA had a diminished DNA binding capacity (measured by electromobility shift analysis) compared to either control or 1,25-D_3 treated extracts. These data demonstrate that phosphorylation has a role in DNA binding of the VDR (Figure 1). Further studies are underway to determine how changes in DNA binding correlate to apoptosis. Luciferase assays are currently being set up to test effects of TPA on transactivation ability of the VDR in MCF-7 cells and the vitamin D_3-resistant variant.

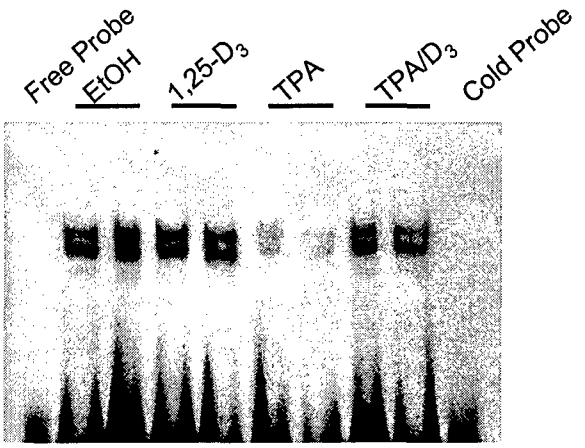

Figure 1 Electromobility shift assay using human p21 VDRE (unpublished data).

Effect of 1,25-D3 on mitochondrial function and caspase activity

In addition to the phosphorylation state of the VDR, we examined the 1,25-D_3 signaling pathway downstream of the VDR in order to identify specific intracellular events involved in 1,25-D_3 mediated apoptosis. These studies will provide baseline data necessary for determining how phosphorylation pathways interact with 1,25-D_3 signaling. Characterization of events, which are blocked in $MCF7^{D3Res}$ cells (a vitamin D_3-resistant variant), is a prerequisite to determining how TPA sensitizes the D_3-resistant cells to 1,25-D_3. In particular, we examined the effects of 1,25-D_3 on mitochondrial function and caspase activity.

Mitochondrial Function

Disruption of mitochondrial function, which is characterized by translocation of Bax to mitochondria, and release of cytochrome *c* into cytosol occurs in the presence of both 1,25-D_3 and TNFα (used as a positive control, undergoes apoptosis by a caspase-dependent mechanism) in MCF-7 cells (Figure 2). Not only was Bax redistributed to mitochondria, but it was also cleaved from 21 kDa to 18 kDa. This observation is consistent with other reports of Bax cleavage during drug-induced apoptosis (5). Bax translocation to mitochondria and apoptosis in response to TNFα can be triggered in $MCF\text{-}7^{D3Res}$ cells indicating that Bax functions appropriately during apoptosis induced by agents other than 1,25-D_3 (Figure 2). The inability of Bax to redistribute to mitochondria may be involved in 1,25-D_3 resistance. Subcellular distribution of Bax in $MCF\text{-}7^{D3Res}$ cells after treatment with TPA±1,25-D_3 will be determined to see if TPA sensitization to 1,25-D_3 effects occurs at this level.

Translocation of Bax to mitochondria is associated with subsequent release of cytochrome *c*, events that are considered to be commitment points for activating apoptosis. 1,25-D_3 induces redistribution of cytochrome *c* from mitochondria to cytosol (Figure 4). This is a first observation implicating a role for mitochondrial events in 1,25-D_3 mediated apoptosis. By contrast, in $MCF\text{-}7^{D3Res}$ cells, TNFα, but not 1,25-D_3, induce release of cytochrome *c* (Figure 4). These experiments will be repeated in $MCF\text{-}7^{D3Res}$ cells in the presence of TPA±1,25-D_3 to determine if TPA sensitization to 1,25-D_3 effects can induce release of cytochrome *c*.

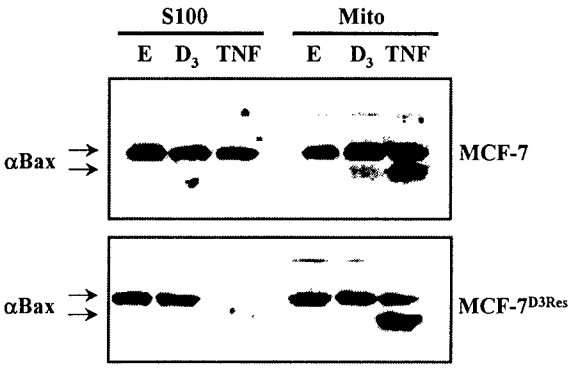

Figure 2 Subcellular distribution of Bax in MCF-7 and MCF-7^{D3Res} cells after treatment with 1,25-D_3 or TNFα (unpublished results).

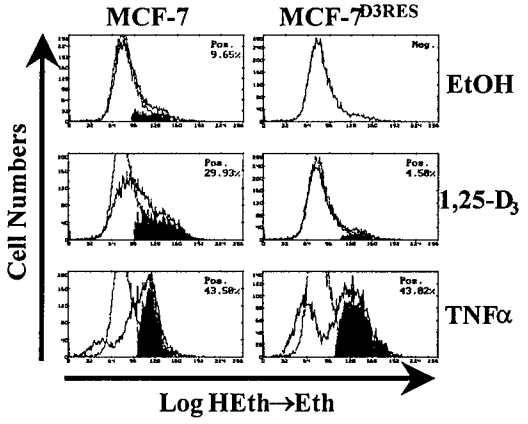

Figure 3 ROS production in MCF-7 or MCF-7^{D3Res} cells after treatment with 1,25-D_3 or TNFα (unpublished results)

Mitochondrial generation of reactive oxygen species (ROS) in response to apoptotic stimuli was examined. Although the source of ROS accounting for apoptosis has not been characterized, disruption of the electron transport chain with release of cytochrome *c*, the ubiquinone complex of the respiratory chain becomes a major source of superoxide radicals. By using flow cytometric techniques, production of superoxide anion was assessed by the degree of oxidation of hydroethidine to ethidium. MCF-7 cells (but not MCF-7^{D3Res} cells) produced ROS in the presence of 1,25-D_3 (Figure 3).

Caspase activity

In order to determine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis, we used a broad-spectrum cell permeable caspase inhibitor (zVAD.fmk) in MCF-7 cells. zVAD.fmk protected MCF-7 cells from TNFα mediated cell death. The caspase inhibitor blocked the effect of TNFα on cell number, ROS production, release of cytochrome *c*, PARP cleavage, phosphatidylserine (PS) externalization, and DNA fragmentation. This demonstrates that TNFα induced cell death requires caspase activation since protease inhibitors can prevent apoptosis. In addition, TNFα induces PARP cleavage in MCF-7^{D3Res} cells indicating that these cells can activate caspases in response to certain apoptotic stimuli (Figure 4).

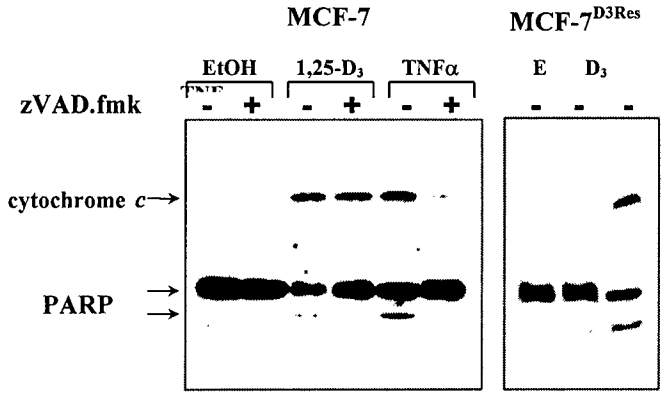

Figure 4 Expression of cytochrome *c* and PARP in MCF-7 cells (in the presence and absence of zVAD.fmk) and MCF-7^{D3Res} cells (unpublished results).

However, zVAD.fmk did not protect MCF-7 cells from 1,25-D_3 mediated apoptosis since no increase in cell number was observed. We further examined the effect of zVAD.fmk on mitochondria and downstream events. The inhibitor had no effect on ROS production or cytochrome *c* release, yet events downstream of mitochondria were blocked such as PARP cleavage, DNA fragmentation, and PS externalization (see Figure 4). This suggests that initiation and induction of 1,25-D_3 mediated apoptosis does not require activation of caspases but the later degradation phase of apoptosis such

as DNA fragmentation may require caspase activity.

CONCLUSION

1,25-D_3 induces apoptosis in MCF-7 cells by disrupting mitochondrial function which is accomplished by translocation of Bax to mitochondria, release of cytochrome *c*, and production of ROS. This is a first observation that demonstrates that 1,25-D_3 signaling on mitochondria does not require caspase activation, since caspase inhibitor was unable to block these events. Although caspase inhibitor was able to block events downstream of mitochondria such as PARP cleavage, external display of PS, and DNA fragmentation, the commitment of MCF-7 cells to 1,25-D_3 mediated cell death is caspase independent. By understanding the effects of 1,25-D_3 on mitochondria and caspase activity, one can determine how TPA (and PKC pathway) interact with vitamin D signaling in potentiating apoptosis in D_3-resistant MCF-7 cells.

This project has important implications for breast cancer since a breast cancer cell that is resistant to the apoptosis-inducing effects of 1,25-D_3 might be sensitized through activators of PKC. This basic knowledge could lead to new therapeutics for treatment of certain forms of breast cancer.

REFERENCES

1. Narvaez CJ and Welsh JW (1997) Differential effects of 1,25-dihydroxyvitamin D_3 and TPA on cell cycle and apoptosis of MCF-7 cells and a vitamin D_3 resistant variant. *Endocrinol.* **138**:4690-4698.
2. Welsh JE, Simboli-Campbell M, Narvaez CJ, and Tenniswood M (1995) Role of apoptosis in the growth inhibitory effects of vitamin D in MCF7 cells. *Adv. Exp. Biol. Med.* **375**:45-52.
3. Xiang J, Chao DT, and Korsmeyer SJ (1996) BAX-induced cell death may not require interleukin 1β-converting enzyme-like proteases. *PNAS* **93**:14559-14563.
4. Susin SA, Zamzami N, and Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim. Biophys. Acta* **1366**:151-165.
5. Wood DE, Thomas A, Devi LA, Berman Y, Reed JC, and Newcomb EW (1998) Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* **17**:1069-78.

APPENDIX A.
Letter indicating report contains unpublished data

University of Notre Dame
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July 22, 1999

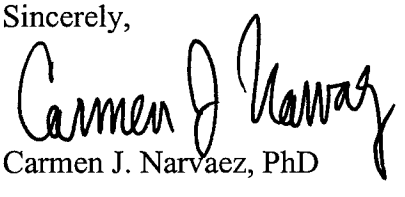
Commander
US Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

Dear Sir or Madam:

Re: Annual Report for "The Role of VDR Phosphorylation in Vitamin D Induced Apoptosis"
DAMD17-97-1-7183

This report contains unpublished data. All the figures contain statements indicating that the data is unpublished. Some of the data has been presented in poster format at a conference, but the data has not been submitted to a peer reviewed journal for publication yet.

Sincerely,



Carmen J. Narvaez, PhD

APPENDIX B.
Key Research Accomplishments

- ✓ First observation implicating a role for mitochondrial events in 1,25-D_3 mediated apoptosis.
- ✓ ***Caspase independent events*** involved in 1,25-D_3 mediated apoptosis.
  - Translocation of Bax
  - Release of cytochrome ^{c}
  - Production of reactive oxygen species
- ✓ ***Caspase dependent events*** involved in 1,25-D_3 mediated apoptosis.
  - PARP cleavage
  - External display of phosphatidylserine
  - DNA fragmentation
- ✓ The observation that 1,25-D_3 mediated cell death is caspase independent.
- ✓ Techniques acquired so far in the course of this study
  - Flow Cytometry
  - Immunoprecipitation
  - Electromobility Shift Assay

APPENDIX C.
Reportable Outcomes

- ✓ Poster presentation at the 1999 Keystone Symposia "**Apoptosis and Programmed Cell Death**" at Breckenridge, CO, April 6-11, 1999. Abstract title: "Caspase-independent apoptosis by vitamin D treatment in MCF-7 breast cancer cells."

- ✓ Awarded a Travel Award for the poster presentation at the 1999 Keystone Symposium.

APPENDIX D.
Copy of cited manuscripts or abstracts

Caspase-Independent Apoptosis By Vitamin D Treatment in MCF-7 Breast Cancer Cells. Carmen J. Narvaez and JoEllen Welsh, Dept of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556

1,25-Dihydroxyvitamin D_3 (1,25-D_3), the active form of vitamin D_3, is not only a powerful regulator of calcium homeostasis, but is a steroid hormone with important roles in cell growth and differentiation. We have shown that 1,25-D_3 induces morphological and biochemical markers of apoptosis (chromatin and cytoplasmic condensation, and DNA fragmentation) in MCF-7 breast cancer cells. In order to probe the mechanisms whereby vitamin D signaling modulates apoptosis in MCF-7 cells, we used a cell permeable inhibitor of caspase-related proteases (zVAD.fmk) to examine the involvement of caspase-dependent proteolysis in 1,25-D_3 mediated apoptosis. The effects of 1,25-D_3 treatment on MCF-7 cells were compared to TNFa which induces apoptosis by a caspase-dependent mechanism. zVAD-fmk protected MCF-7 cells from TNFa mediated apoptosis but did not protect MCF-7 cells from the effects of 1,25-D_3 treatment. The caspase inhibitor did not block the effects of 1,25-D_3 on cell number, mitochondrial membrane potential, or release of cytochrome c. zVAD-fmk did prevent 1,25-D_3 mediated DNA fragmentation, PARP cleavage, and external display of phosphatidylserine (PS). These studies suggest that the 1,25-D_3 mediated apoptotic events occurring downstream of mitochondrial disruption are blocked by caspase inhibition, but the commitment of MCF-7 cells to 1,25-D_3 mediated apoptosis is caspase independent. *Supported by NIH (#CA69700) & DAMD (#17-7-1-7183).*

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FOR THE COMMANDER:

PHYLLIS M. ORINEHART
Deputy Chief of Staff for
Information Management